



Tuberculous Lymphadenitis Is Associated with Enhanced Baseline and Antigen-Specific Induction of Type 1 and Type 17 Cytokines and Reduced Interleukin-1 β (IL-1 β) and IL-18 at the Site of Infection

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ABSTRACT Tuberculous lymphadenitis (TBL) is characterized by an expansion of Th1 and Th17 cells with altered serum levels of proinflammatory cytokines. However, the cytokine profile at the site of infection, i.e., the affected lymph nodes, has not been examined in detail. To estimate the baseline and mycobacterial antigen-stimulated concentrations of type 1, type 17, and other proinflammatory cytokines in patients with TBL ($n = 14$), we examined both the baseline and the antigen-specific concentrations of these cytokines before and after chemotherapy and compared them with those in individuals with pulmonary tuberculosis (PTB) ($n = 14$). In addition, we also compared the cytokine responses in whole blood and those in the lymph nodes of TBL individuals. We observed significantly enhanced baseline and antigen-specific levels of type 1 cytokines (gamma interferon [IFN- γ] and tumor necrosis factor alpha [TNF- α]) and a type 17 cytokine (interleukin-17 [IL-17]) and significantly diminished baseline and antigen-specific levels of proinflammatory cytokines (IL-1 β and IL-18) in the whole blood of TBL individuals compared to those in the whole blood of PTB individuals. Moreover, we also observed a pattern of baseline and antigen-specific cytokine production at the site of infection (lymph node) similar to that in the whole blood of TBL individuals. Following standard antituberculosis (anti-TB) treatment, we observed alterations in the baseline and/or antigen-specific levels of IFN- γ , TNF- α , IL-1 β , and IL-18. TBL is therefore characterized by enhanced baseline and antigen-specific production of type 1 and type 17 cytokines and reduced baseline and antigen-specific production of IL-1 β and IL-18 at the site of infection.

KEYWORDS tuberculosis, lymphadenitis, cytokines

Immune responses in tuberculous lymphadenitis (TBL) are poorly understood, although TBL is the most common form of extrapulmonary TB, accounting for 30 to 40% of cases (1). TBL commonly affects the superficial lymph nodes (LN) of the body, with the cervical lymph nodes being the most commonly affected, followed by the inguinal, axillary, mesenteric, mediastinal, and intramammary lymph nodes (2). The pathogenesis of TBL and/or the pathway of dissemination from the lungs is still unclear,

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TABLE 1 Demographic characteristics and hematological profiles of the study population

Characteristic	Value(s) for the following individuals:				P value ^a
	TBL			PTB	
	Pretreatment	Posttreatment	Total		
Demographic characteristics					
No. of individuals recruited	14	14		14	
Gender (no. of M/no. of F ^b)	4/10	4/10		13/1	
Median (range) age (yr)	30 (18–47)	30 (18–47)		39 (26–60)	
No. of individuals with culture grade 0/1+/2+/3+	4/9/1/0			2/8/3/1	
No. of individuals with smear grade 0/1+/2+/3+	10/3/1/0			3/10/1/0	
Hematological profile ^c					
No. of whole blood cells (10 ³ /liter)			7.95 (3.8–10.9)	7.97 (6.2–13.2)	NS
No. of red blood cells (10 ⁶ /liter)			4.86 (3.94–5.72)	4.14 (3.60–5.90)	NS
Lymphocytes (%)			29.6 (22.9–41.5)	20.5 (13.0–37.0)	0.0075
Neutrophils (%)			57.2 (45.8–66.4)	59.1 (44.0–75.0)	0.0248
No. of platelets (10 ³ /liter)			368.4 (199–654)	346.5 (249–633)	NS
Hemoglobin concn (g/dl)			12.55 (10.5–16.0)	11.8 (9.0–16.9)	NS

^aCalculated using the Mann-Whitney U test. NS, not significant.

^bM, males; F, females.

^cData represent the median (range).

although it is proposed to occur by hematogenous spread (2). In women and children, TBL is much more common than pulmonary tuberculosis (PTB) (3).

Previous studies have shown that TBL is characterized by a mycobacterial antigen-specific expansion of mono- and multifunctional Th1 and Th17 cells, as well as increased frequencies of CD8⁺ T cells expressing type 1 and type 17 cytokines in comparison to those in individuals with PTB (4, 5). In contrast, TBL is associated with reduced systemic and antigen-stimulated production of certain proinflammatory cytokines, including interleukin-1 β (IL-1 β) and IL-18 (6), and elevated systemic levels of others, including IL-8 and tumor necrosis factor beta (TNF- β) (7). Since type 1, type 17, and other proinflammatory cytokine responses are classically postulated to be associated with protective immunity (8), it is possible that the reduction in IL-1 β and IL-18 levels reflects a failure of TBL individuals to mount adequate protective immunity. Moreover, the nature of the cytokine response at the site of infection in TBL individuals (the affected lymph node) has not been examined in detail. In children with TBL, it was shown that low numbers of CD8⁺ T cells correlated with high levels of transforming growth factor β , IL-13, and FoxP3-positive regulatory T cells in the affected lymph nodes (9). In addition, higher number of cells expressing tumor necrosis factor alpha (TNF- α) have also been shown to be present in the lymph nodes of individuals with TBL (10, 11). However, the antigen-stimulated production of cytokines was not assessed at the site of infection in any of these studies.

Hence, we wanted to characterize the antigen-specific cytokine profile of the immune responses in the peripheral blood of TBL individuals compared to that in the peripheral blood of PTB individuals as well as to compare the cytokine responses in whole blood versus that in the lymph nodes of TBL individuals. We show that TBL is predominantly characterized by enhanced antigen-specific type 1 and type 17 cytokine responses and reduced antigen-specific production of IL-1 β and IL-18 both in the periphery (in comparison to that in PTB individuals) and at the site of infection (in comparison to that in whole blood). Most of these responses were not significantly reversed following standard antituberculosis treatment.

RESULTS

Study population characteristics. The baseline characteristics of TBL and PTB individuals, including demographic and hematological features, are shown in Table 1. The smear and culture grades for TBL represent those of lymph node smears and culture, respectively, while the smear and culture grades for PTB represent those of sputum smears and culture, respectively. As shown in Table 1, TBL individuals exhibited

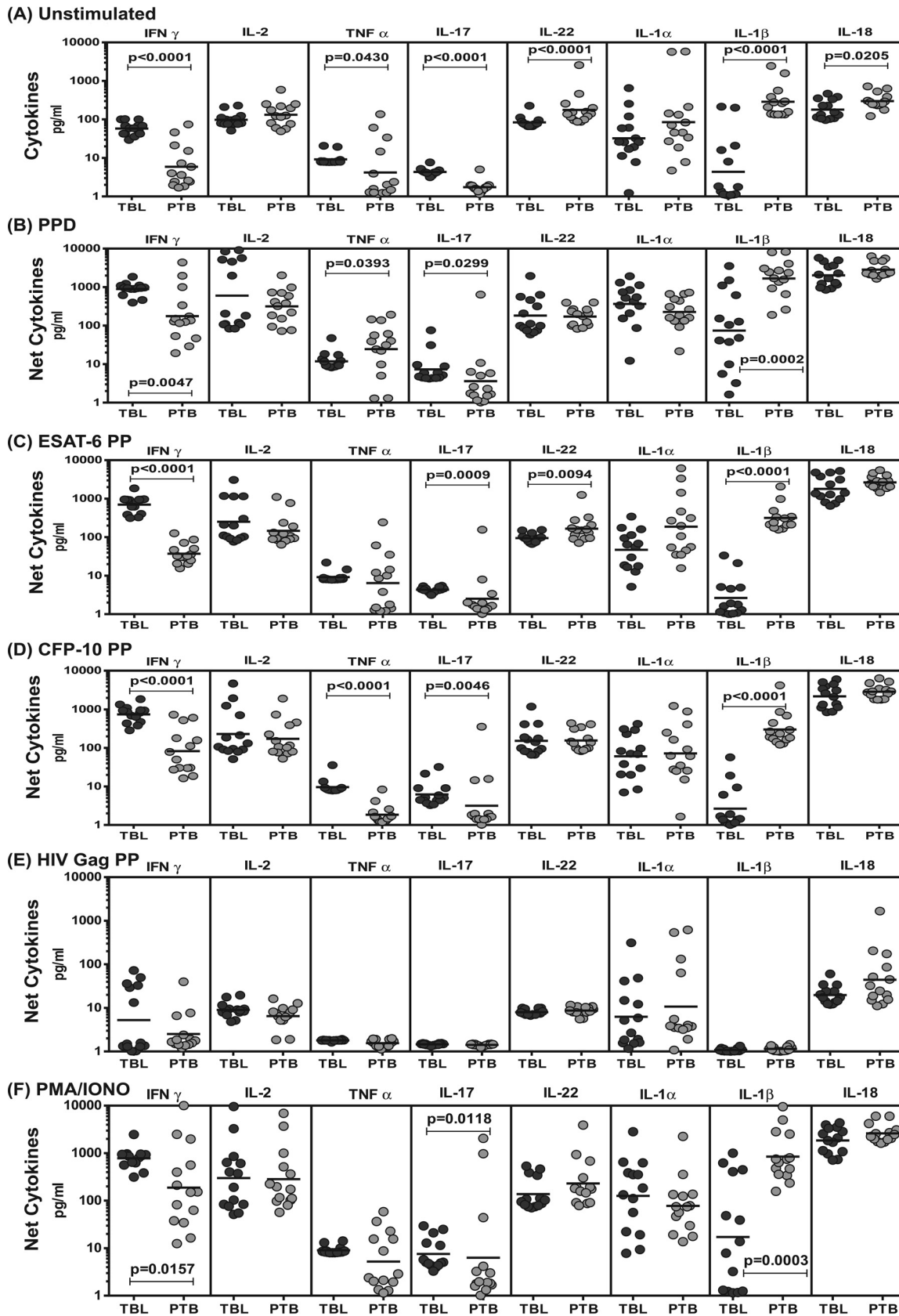


FIG 1 TBL is associated with increased baseline and antigen-specific levels of type 1 and type 17 cytokines and decreased baseline and antigen-specific levels of IL-1 β and IL-18 in comparison to those found in PTB. Whole blood from TBL ($n = 14$) and PTB ($n = 14$) individuals was cultured with medium alone or mycobacterial or control antigens for 18 h, and the baseline and antigen-stimulated levels of type

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significantly higher percentages of lymphocytes but significantly lower percentages of neutrophils than PTB individuals.

TBL is associated with enhanced baseline and antigen-specific levels of type 1 and type 17 cytokines and reduced baseline and antigen-specific levels of IL-1 β and IL-18 in comparison to those during PTB. To determine the antigen-specific cytokine profile in TBL individuals in comparison to that in PTB individuals, we measured the baseline and antigen-stimulated levels of type 1 cytokines (gamma interferon [IFN- γ], TNF- α , and IL-2), type 17 cytokines (IL-17 and IL-22), and other proinflammatory cytokines (IL-1 α , IL-1 β , and IL-18) in TBL and PTB individuals following stimulation of whole blood with mycobacterial and other antigens (Fig. 1). As shown in Fig. 1A, TBL individuals exhibited significantly increased levels of IFN- γ , TNF- α , and IL-17 and significantly decreased levels of IL-22, IL-1 β , and IL-18 at baseline in comparison to PTB individuals. Similarly, as shown in Fig. 1B to D, TBL individuals exhibited significantly increased levels of purified protein derivative (PPD)-, early secreted antigen 6 (ESAT-6)-, and culture filtrate protein 10 (CFP-10)-induced levels of IFN- γ , TNF- α (except in response to ESAT-6), and IL-17 and significantly decreased levels of IL-1 β in comparison to PTB individuals. However, TBL individuals exhibited no significant differences in the levels of any of the cytokines in response to the control antigen, which was a human immunodeficiency virus Gag peptide pool (HIVPP) (Fig. 1E). Finally, as shown in Fig. 1F, TBL individuals exhibited significantly increased levels of IFN- γ and IL-17 and significantly decreased levels of IL-1 β in response to phorbol 12-myristate 13-acetate (PMA)-ionomycin (PMA-Iono). Thus, TBL is associated with enhanced levels of certain type 1 and type 17 cytokines and diminished levels of IL-1 β and IL-18 in comparison to PTB.

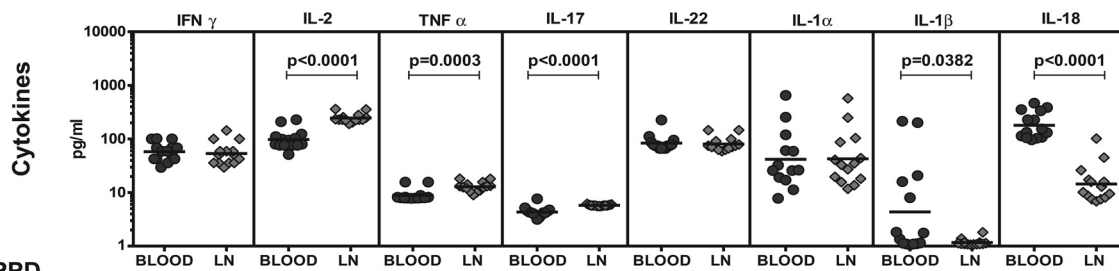
TBL is associated with enhanced baseline and antigen-specific levels of type 1 and type 17 cytokines and reduced baseline and antigen-specific levels of IL-1 β and IL-18 at the site of infection. To determine the site-specific cytokine profile in the lymph nodes versus the whole blood of TBL individuals, we measured the baseline and antigen-stimulated levels of type 1 cytokines (IFN- γ , TNF- α , and IL-2), type 17 cytokines (IL-17 and IL-22), and other proinflammatory cytokines (IL-1 α , IL-1 β , and IL-18) in whole blood and the affected lymph node of TBL individuals (Fig. 2). As shown in Fig. 2A, at baseline, TBL individuals exhibited significantly increased levels of IL-2, TNF- α , and IL-17 as well as significantly decreased levels of IL-1 β and IL-18 in lymph nodes in comparison to those in whole blood. Similarly, as shown in Fig. 2B to D, TBL individuals exhibited significantly increased levels of PPD-, ESAT-6-, and CFP-10-induced TNF- α and IL-17 and significantly decreased levels of IL-1 β and IL-18 in lymph nodes compared to those in whole blood. In addition, TBL individuals also exhibited significantly decreased levels of IL-1 α (in response to PPD) and IL-22 (in response to ESAT-6) in lymph nodes. However, TBL individuals exhibited no significant differences in the levels of any of the cytokines in response to the control antigen (HIVPP) between lymph nodes and whole blood (Fig. 2E). Finally, as shown in Fig. 2F, TBL individuals exhibited significantly increased levels of IL-2, TNF- α , and IL-17 and significantly decreased levels of IL-1 α , IL-1 β , and IL-18 in response to PMA-Iono in lymph nodes compared to those in whole blood. Thus, our analysis of LN samples expands the data in Fig. 1, showing that TBL is associated with focused responses of type 1 and type 17 cytokines and diminished levels of IL-1 β and IL-18.

Treatment of TBL is associated with increased systemic concentrations of IL-1 β and IL-18. To determine the effect of treatment on the cytokine profile in TBL individuals,

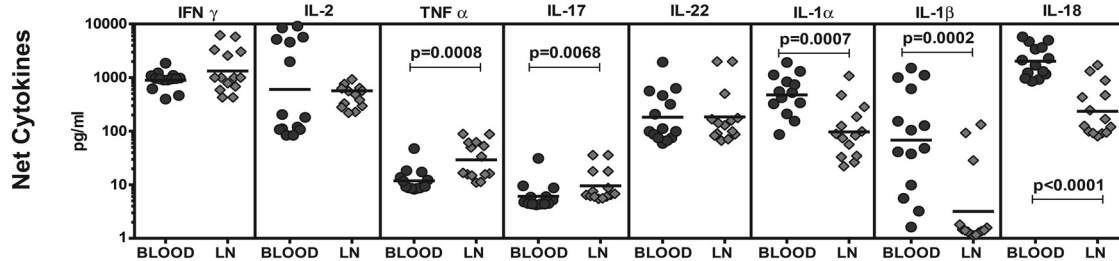
FIG 1 Legend (Continued)

1, type 17, and other proinflammatory cytokines were measured. The baseline levels (A) as well as the levels of type 1 cytokines (IFN- γ , IL-2, and TNF- α), type 17 cytokines (IL-17 and IL-22), and other proinflammatory cytokines (IL-1 α , IL-1 β , and IL-18) stimulated by PPD (B), ESAT-6 peptide pools (C), CFP-10 peptide pools (D), HIV Gag peptide pools (E), and PMA-Iono (F) in TBL and PTB individuals are shown. Each circle represents a single individual, and the bars represent the geometric mean values. Net cytokine levels were calculated by subtracting the baseline levels from the antigen-induced levels for each individual. *P* values were calculated using the Mann-Whitney U test.

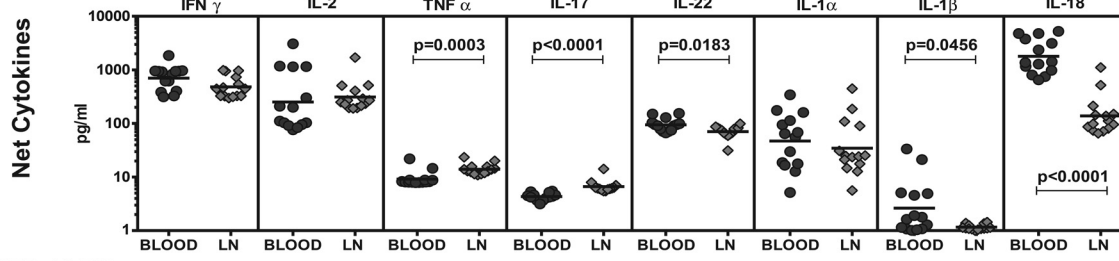
(A) Unstimulated



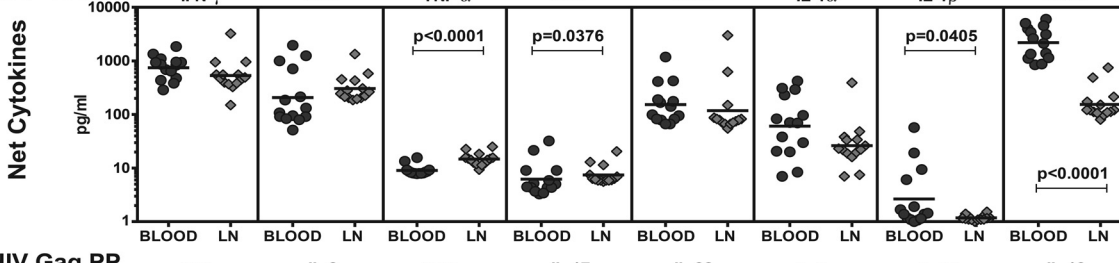
(B) PPD



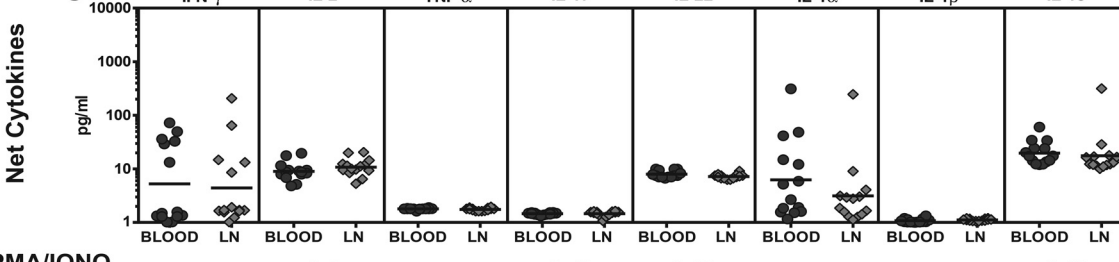
(C) ESAT-6 PP



(D) CFP-10 PP



(E) HIV Gag PP



(F) PMA/IONO

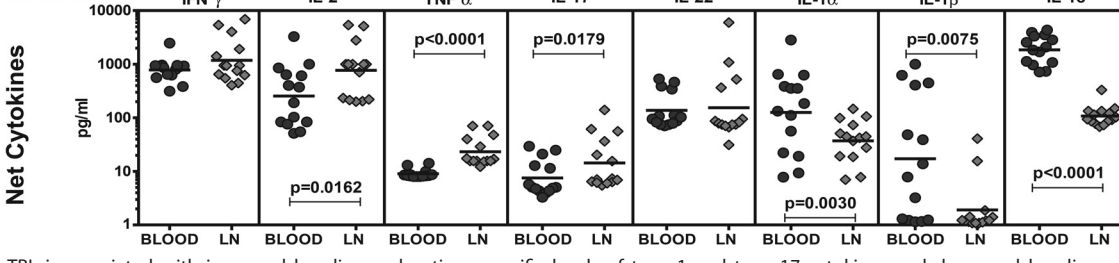


FIG 2 TBL is associated with increased baseline and antigen-specific levels of type 1 and type 17 cytokines and decreased baseline and antigen-specific levels of IL-1β and IL-18 at the site of infection. Whole blood and lymph node cells from TBL (*n* = 14) individuals were cultured with medium alone or mycobacterial or control antigens for 18 h, and the baseline and antigen-stimulated levels of type 1, type 17, and other

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we measured the baseline and antigen-specific levels of type 1 cytokines (IFN- γ , TNF- α , and IL-2), type 17 cytokines (IL-17 and IL-22), and other proinflammatory cytokines (IL-1 α , IL-1 β , and IL-18) in TBL individuals before treatment and after treatment (Fig. 3). As shown in Fig. 3A, TBL individuals exhibited significantly increased baseline levels of IL-1 β and IL-18 but not the other cytokines at posttreatment time points compared to those at pretreatment time points. Similarly, as shown in Fig. 3B to D, TBL individuals exhibited significantly decreased levels of IFN- γ (in response to PPD) and significantly increased levels of TNF- α (in response to PPD) and IL-1 β (in response to PPD, ESAT-6, and CFP-10) at posttreatment time points compared to those at pretreatment time points. While TBL individuals exhibited no significant differences in the levels of any of the cytokines in response to HIVPP at posttreatment time points (Fig. 3E), they exhibited significantly increased levels of IL-1 β in response to PMA-Iono at posttreatment time points compared to those at pretreatment time points (Fig. 3F). Thus, treatment of TBL is associated with moderate alterations in the baseline and antigen-specific levels of cytokines.

Mycobacterial antigens stimulate increased cytokine production from peripheral blood of TBL individuals. To determine the mycobacterial antigen-stimulated level of cytokines in TBL individuals, we measured the mycobacterial antigen-stimulated levels of type 1 cytokines (IFN- γ , TNF- α , and IL-2), type 17 cytokines (IL-17 and IL-22), and other proinflammatory cytokines (IL-1 α , IL-1 β , and IL-18) in the peripheral blood of TBL individuals following stimulation of whole blood with PPD and other antigens (Fig. 4). As shown, PPD (Fig. 4A), ESAT-6 (Fig. 4B), CFP-10 (Fig. 4C), HIV Gag peptides (Fig. 4D), and PMA-Iono (Fig. 4E) all induced significantly elevated levels of IFN- γ , IL-2, TNF- α , IL-17, IL-22, IL-1 α , IL-1 β , and IL-18 in TBL individuals.

Mycobacterial antigens stimulate increased cytokine production from lymph node cells of TBL individuals. To determine the mycobacterial antigen-stimulated level of cytokines at the site of infection in TBL individuals, we measured the mycobacterial antigen-stimulated levels of type 1 cytokines (IFN- γ , TNF- α , and IL-2), type 17 cytokines (IL-17 and IL-22), and other proinflammatory cytokines (IL-1 α , IL-1 β , and IL-18) in TBL individuals following stimulation of lymph node tissue cultures with PPD and other antigens (Fig. 5). As shown, PPD (Fig. 5A), ESAT-6 (Fig. 5B), CFP-10 (Fig. 5C), HIV Gag peptides (Fig. 5D), and PMA-Iono (Fig. 5E) all induced significantly elevated levels of IFN- γ , IL-2, TNF- α , IL-17, IL-22, IL-1 α , IL-1 β , and IL-18 in TBL individuals.

DISCUSSION

Type 1, type 17, and other proinflammatory cytokines are known to play protective roles in both animal models of *Mycobacterium tuberculosis* infection and human infection (12), while type 1 interferons and regulatory cytokines promote susceptibility (8, 13). The roles of cytokines in extrapulmonary forms of tuberculosis (TB) are poorly understood. We have previously shown that tuberculous lymphadenitis is associated with an expansion of mono- and multifunctional Th1 and Th17 cells and with reduced systemic levels of IL-1 β and IL-18 (4, 6). We expanded on those findings in this study by performing an analysis of antigen-induced cytokine responses in the whole blood of TBL and PTB individuals. We also performed a comprehensive comparison of the cytokine responses between whole blood and the affected lymph node within TBL individuals. Finally, we also examined the modulation of the peripheral cytokine responses in TBL individuals after curative therapy.

Our data obtained by comparison of the baseline and antigen-stimulated cytokine responses in TBL and PTB individuals reveal the following salient features. First, the

FIG 2 Legend (Continued)

proinflammatory cytokines were measured. The baseline levels (A) as well as the levels of type 1 cytokines (IFN- γ , IL-2, and TNF- α), type 17 cytokines (IL-17 and IL-22), and other proinflammatory cytokines (IL-1 α , IL-1 β , and IL-18) stimulated by PPD (B), ESAT-6 peptide pools (C), CFP-10 peptide pools (D), HIV Gag peptide pools (E), and PMA-Iono (F) in whole blood and lymph node cell cultures from TBL individuals are shown. Each circle or diamond represents a single individual, and the bars represent the geometric mean values. Net cytokine levels were calculated by subtracting the baseline levels from the antigen-induced levels for each individual. *P* values were calculated using the Mann-Whitney U test.

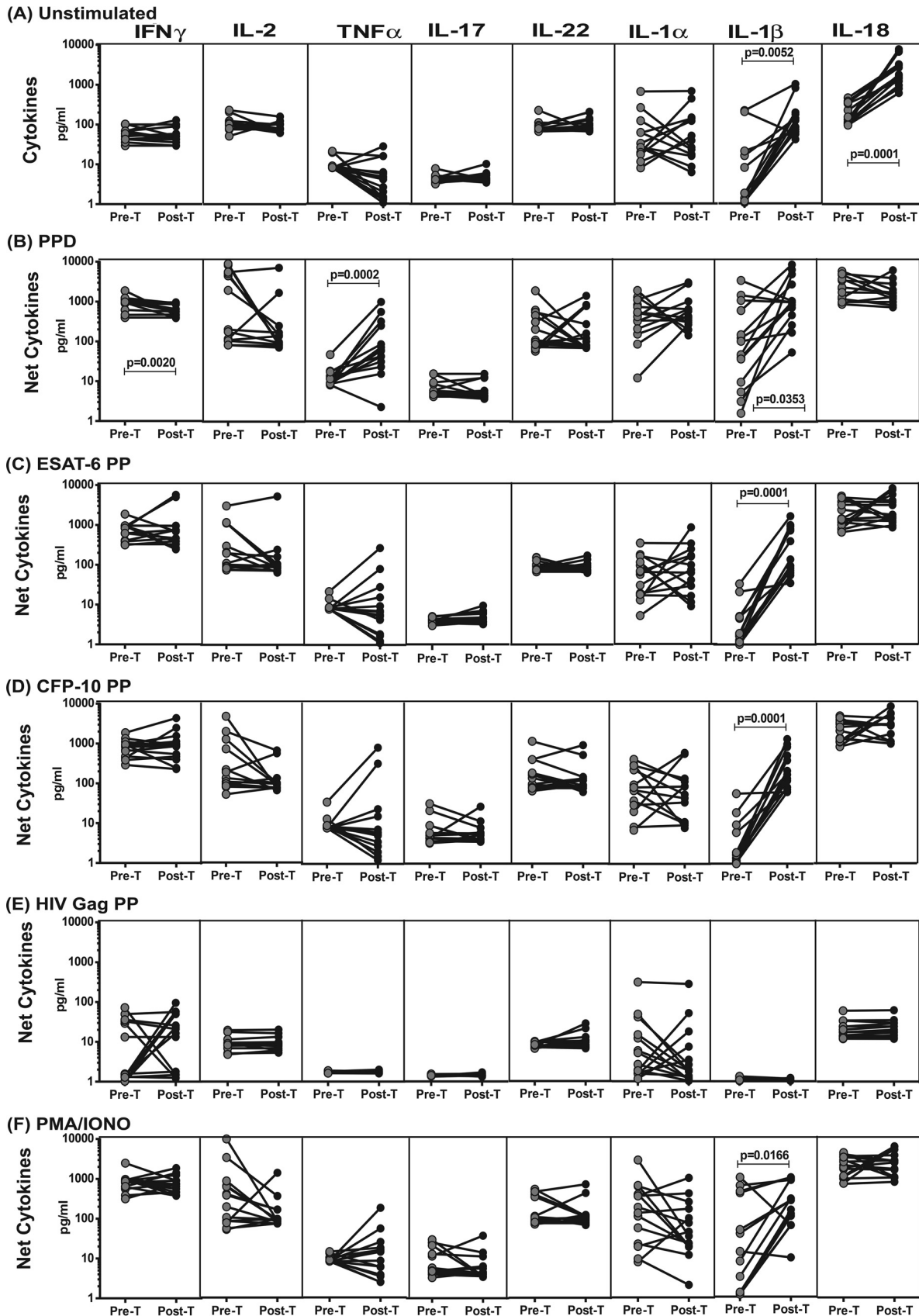


FIG 3 Treatment modifies the baseline and antigen-specific cytokine profile in TBL individuals. Whole blood from TBL individuals ($n = 14$) collected before antituberculosis therapy (pre-T) and after antituberculosis therapy (post-T) was cultured with medium alone or mycobacterial (Continued on next page)

differences in peripheral immune responses are manifest at the baseline, since the augmentation of type 1 and type 17 cytokines and the diminution of IL-1 β and IL-18 are present even under unstimulated conditions. This suggests that although TBL is typically a paucibacillary disease, it is associated with a more pronounced systemic proinflammatory environment (at least in the context of type 1 and type 17 cytokines) than PTB is. The cause of this differential response needs further exploration. Second, IL-22 behaves differently from IL-17, in that the induction is significantly lower in TBL individuals than PTB individuals. This could perhaps be due to the role of IL-22 as a mucosal protective cytokine (14), and therefore, IL-22 might have a more prominent role in PTB than in TBL. Third, our data corroborate our previous finding of enhanced Th1 and Th17 responses in TBL individuals (4) by demonstrating increased expression of the cytokines upon whole blood stimulation as well. Fourth, our findings of reduced antigen-specific levels of IL-1 β and IL-18 in TBL individuals than in individuals with latent *M. tuberculosis* infection (6) is herein demonstrated to extend to PTB as well, reinforcing the notion that these cytokines might serve as important correlates of protective immunity in TBL. Fifth, all three antigen/peptide pools (PP) used for mycobacterial antigen stimulation (PPD, ESAT-6 PP, and CFP-10 PP) showed remarkably consistent findings. Sixth, the differential cytokine response did not extend to bystander control antigens since HIV peptide pools did not demonstrate any cytokine response (differential or otherwise). Seventh, some of these differential cytokine responses might be intrinsic to the cell since PMA-Iono stimulation induced changes similar to those induced by mycobacterial antigens. Finally, only some and not most of these cytokine responses were modulated by antituberculosis treatment.

The major highlight of our study was the examination of cellular immune responses at the site of infection, i.e., the affected lymph nodes. Previous studies have shown that *M. tuberculosis* infection is associated with an altered cellular composition in infected lymph nodes, with few CD8⁺ cytotoxic T cells and increased numbers of regulatory T cells being found (9). However, almost all of the cytokine responses have been examined by the use of either mRNA expression or immunohistochemistry (9–11). To our knowledge, our study is the first to examine the antigen-specific cytokine responses in the affected lymph nodes. Our data reveal that the cytokine profile at the site of infection is characterized by enhanced type 1 and type 17 cytokine responses and depressed IL-1 β and IL-18 responses. The latter finding reinforces our previous observation that diminution of the IL-1 β and IL-18 responses could potentially be associated with increased susceptibility to TBL and therefore serve as a correlate of protective immunity in this form of extrapulmonary TB. However, the lack of compromise in the production of type 1 and type 17 cytokines also suggests that these are not the only correlates of protective immunity in TBL. We propose that the changes in the cytokine profile in TBL individuals both reflect pathways of pathogenicity and provide associations for correlates of protective immunity. The complete profile of cellular and cytokine responses in the affected lymph nodes needs to be explored in the future.

Our study suffers from certain limitations, including a small sample size, a lack of control lymph nodes, etc. Nevertheless, our data still clearly highlight certain pathways associated with the pathology of tuberculous lymphadenitis. Future examination of these pathways in more detail should shed more light on the pathogenesis of this poorly studied manifestation of TB and provide clues to come up with newer modalities to alter susceptibility to this common form of *M. tuberculosis* disease.

FIG 3 Legend (Continued)

or control antigens for 18 h, and the baseline and antigen-stimulated levels of type 1, type 17, and other proinflammatory cytokines were measured. The baseline levels (A) as well as the levels of type 1 cytokines (IFN- γ , IL-2, and TNF- α), type 17 cytokines (IL-17 and IL-22), and other proinflammatory cytokines (IL-1 α , IL-1 β , and IL-18) stimulated by PPD (B), ESAT-6 peptide pools (C), CFP-10 peptide pools (D), HIV Gag peptide pools (E), and PMA-Iono (F) in TBL individuals pre- and posttreatment are shown. Each line represents a single individual. Net cytokine levels were calculated by subtracting the baseline levels from the antigen-induced levels for each individual. *P* values were calculated using the Mann-Whitney U test.

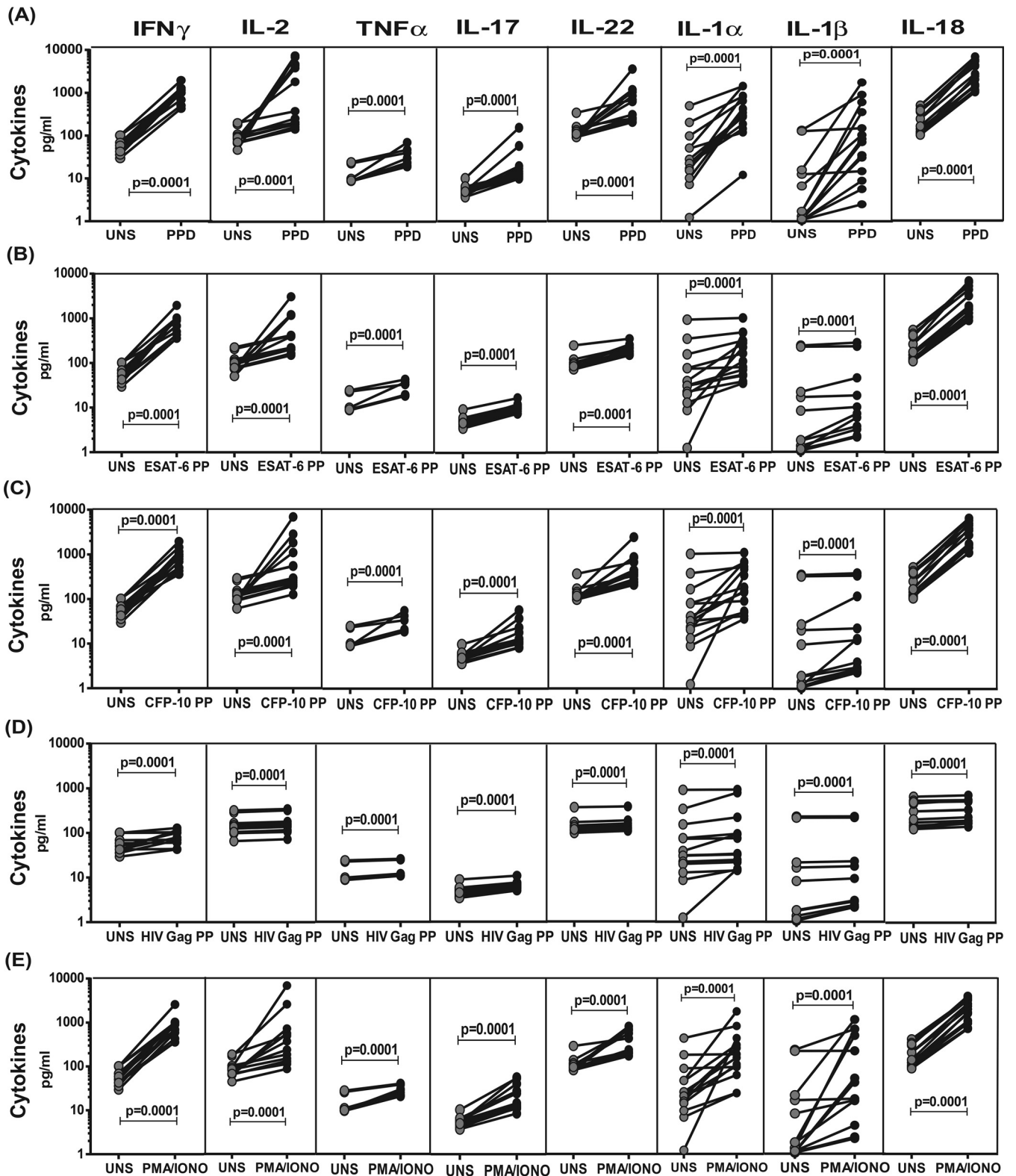


FIG 4 Mycobacterial antigen stimulated increased levels of cytokines in the peripheral blood of TBL individuals. Whole blood from TBL individuals ($n = 14$) was cultured with medium alone or mycobacterial or control antigens for 18 h, and the baseline and antigen-stimulated levels of type 1, type 17, and other proinflammatory cytokines were measured. The levels of type 1 cytokines (IFN- γ , IL-2, and TNF- α), type 17 cytokines (IL-17 and IL-22), and other proinflammatory cytokines (IL-1 α , IL-1 β , and IL-18) stimulated by PPD (A), ESAT-6 peptide pools (B), CFP-10 peptide pools (C), HIV Gag peptide pools (D), and PMA-Iono (E) in TBL individuals are shown. Each line represents a single individual. *P* values were calculated using the Wilcoxon signed-rank test. UNS, unstimulated.

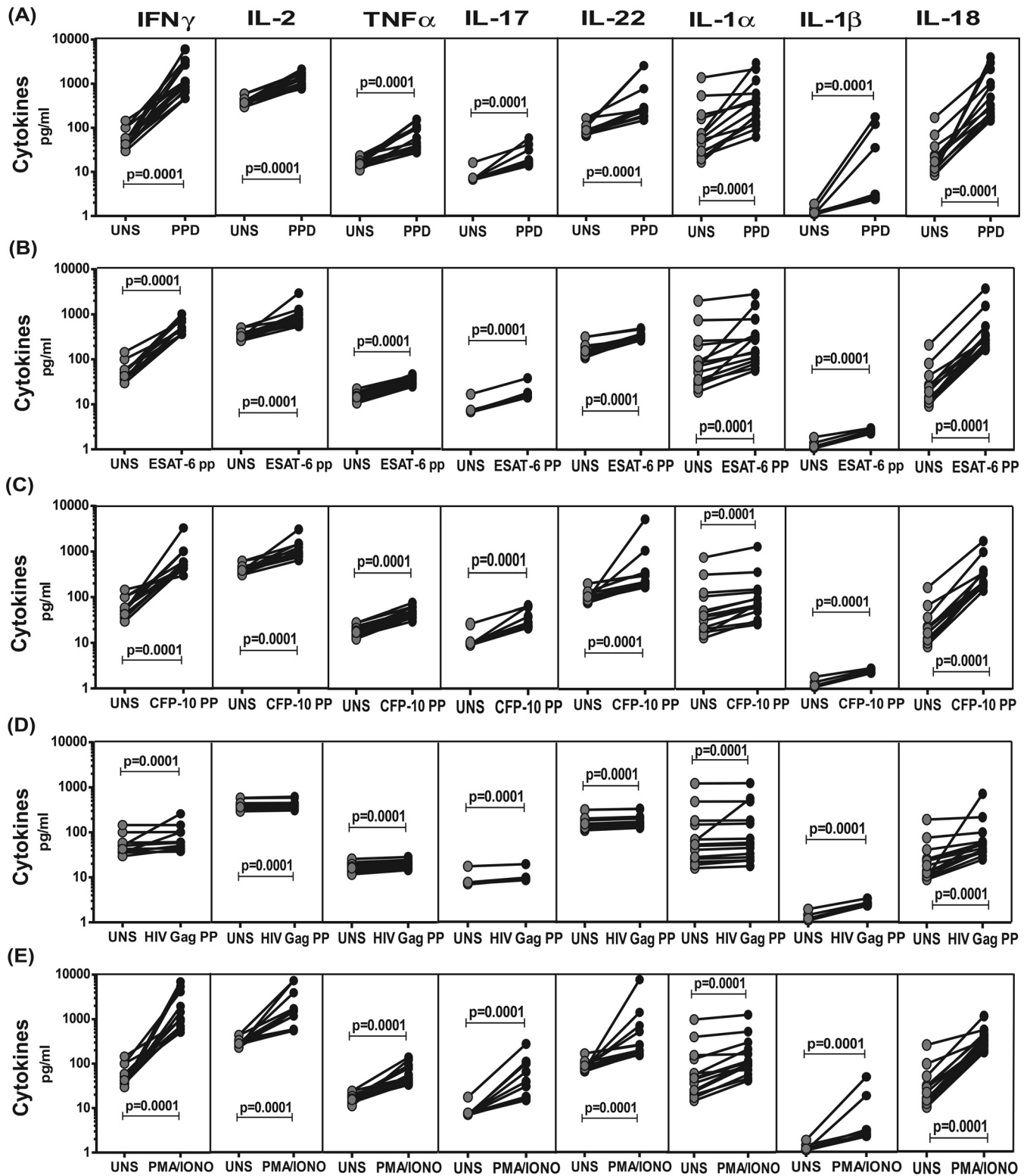


FIG 5 Mycobacterial antigen stimulated increased levels of cytokines in cultures of lymph node tissue from TBL individuals. Lymph node cells from TBL individuals ($n = 14$) were cultured with medium alone or mycobacterial or control antigens for 18 h, and the baseline and antigen-stimulated levels of type 1, type 17, and other proinflammatory cytokines were measured. The levels of type 1 cytokines (IFN- γ , IL-2, and TNF- α), type 17 cytokines (IL-17 and IL-22), and other proinflammatory cytokines (IL-1 α , IL-1 β , and IL-18) stimulated by PPD (A), ESAT-6 peptide pools (B), CFP-10 peptide pools (C), HIV Gag peptide pools (D), and PMA-Iono (E) in TBL individuals are shown. Each line represents a single individual. P values were calculated using the Wilcoxon signed-rank test.

MATERIALS AND METHODS

Ethics statement. This study was specifically approved by the Institutional Ethics Committee of the National Institute for Research in Tuberculosis, and informed written consent was obtained from all participants.

Study population. We studied a group of 14 individuals with TBL and 14 individuals with PTB (Table 1). TBL was diagnosed on the basis of an excision biopsy specimen of the affected lymph node showing either culture positivity or a histopathology confirming the presence of *Mycobacterium tuberculosis*. The lymph node excision biopsy was performed purely for diagnostic purposes. Thus, patients suspected of having TBL consented to undergo excision biopsy. PTB was diagnosed on the basis of sputum culture positivity for *M. tuberculosis* in solid medium culture. All individuals were HIV negative. These individuals are representative of the infected individuals in our study population in terms of their clinical and radiological presentations. The individuals were not on any steroid treatment. Baseline blood samples were collected before any anti-TB medication was administered. All TBL individuals were administered standard anti-TB treatment for 6 months, and at the end of treatment, blood was again collected from these individuals.

Hematological parameters. The leukocyte and differential counts (Table 1) for the study participants were also performed using an ACT-5 Diff hematology analyzer (Beckman Coulter).

Antigens. Mycobacterial antigens, including purified protein derivative (PPD; Statens Serum Institute) and recombinant early secreted antigen 6 (ESAT-6) peptide pools and culture filtrate protein 10 (CFP-10) peptide pools (both peptide pools were from BEI Resources, National Institute of Allergy and Infectious Diseases [NIAID], National Institutes of Health [NIH]), were used as the antigenic stimuli. In addition, a human immunodeficiency virus Gag peptide pool (HIVPP; AIDS Reagent Program, Division of AIDS, NIAID, NIH) was used as a negative control. The final concentrations of antigens were 10 μ g/ml for PPD and 10 μ g/ml for each of the ESAT-6, CFP-10, and HIV Gag peptide pools. Phorbol 12-myristate 13-acetate (PMA)-ionomycin (PMA-Iono; Calbiochem) was used as a positive-control stimulus at final concentrations of 12.5 ng/ml and 125 ng/ml. Dose-response studies have been performed previously, and the optimal concentrations were derived from the previous studies (4, 5).

In vitro whole-blood culture. The supernatants of the *in vitro* cultures of whole blood cells and lymph node tissue were used to measure the cytokine responses. Initially, whole blood was diluted 1:1 with RPMI 1640 medium supplemented with penicillin-streptomycin (100 U/100 mg/ml), L-glutamine (2 mM), and HEPES (10 mM) (Invitrogen, San Diego, CA) and equally dispersed (2 ml/well) in 12-well tissue culture plates (Costar; Corning Inc., Corning, NY). Likewise, dissected lymph nodes (LN) were harvested in RPMI 1640 medium and immediately processed. Each LN was washed twice and chopped into smaller pieces in serum-free RPMI 1640 medium. The chopped LN was then treated with Liberase (0.1 mg/ml) and DNase (0.1 mg/ml) enzymes (Roche Diagnostics) and incubated for 20 to 30 min. After incubation, the cells were washed with RPMI 1640 medium and centrifuged at 2,600 rpm for 10 min. The supernatant was discarded, and the cells were further used for stimulation. The cultures were stimulated with PPD, ESAT-6, CFP-10, HIVPP, or PMA-Iono or were left unstimulated. Subsequently, the costimulatory molecules CD49d/CD28 were added (for whole-blood culture), and the culture was incubated at 37°C in 5% CO₂ for 18 h. Similarly, FastImmune brefeldin A solution (10 μ g/ml) was added to the cultures after 2 h. After 18 h, the cells were transferred to sterile 50-ml Falcon tubes, and centrifugation was done. The culture supernatants were transferred to 2-ml screw-cap tubes and stored at -80°C. The optimal time point for cytokine measurement was determined from previous studies (12).

ELISA. The supernatants of the whole-blood and lymph node tissue cultures were used in the experiments. The cytokine and chemokine (IFN- γ , TNF- α , IL-1 α , IL-1 β , IL-2, IL-17A, IL-18, and IL-22) levels were measured using DuoSet enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems.

Statistics. The data analyses were performed using GraphPad Prism (version 6) software (GraphPad Software Inc., San Diego, CA). The geometric means (GM) were used as measurements of the central tendency. Statistically significant differences between two groups were analyzed using the nonparametric Mann-Whitney U test. The Wilcoxon signed-rank test was used to compare the levels obtained pre- and posttreatment and the levels obtained without stimulation and with stimulation with mycobacterial or other antigens.

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